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ARTICLE

EARTHWORM INFUSION AS AN ANTIMICROBIAL AGAINST BACTERIA CAUSING DIARRHEA

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ABSTRACT

Diarrhea is the second most common disease worldwide after Acute Respiratory Infection (ARI). One of the causes of diarrhea is the gastrointestinal infection caused by disease bacteria (microbe) such as *Salmonella sp., Escherichia coli, Shigella sp.,* and *Vibrio cholerae*. The earthworm species *Lubricus sp* can be used as an alternative medication believed to be able to cure digestive diseases such as typhus, dysentery, and other indigestion like gastritis. This research is laboratory experimental research. The research was conducted in the microbiology laboratory of the medical faculty, Universitas Muhammadiyah Yogyakarta. The antibacterial activity is measured by determining the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values of earthworm infusion *(Lumbricus rubellus)* using the tube dilution test. The bacteria used in this research are *Escherichia coli, Shigella dysentriae,* and *Vibrio cholerae,* isolated from the feces of diarrhea patients in Mutilan Regional General Hospital. This research aims to examine the earthworm infusion antibacterial *(Lumbricus rubellus)* activity to several bacteria that commonly cause diarrhea. According to the study's findings, the average minimum inhibitory concentration (MIC) of earthworm infusion (Lumbricus rubellus) against Escherichia coli, Shigella dysentriae, and Vibrio cholerae is greater than 50%. When it comes to Shigella dysentriae, the average MBC value of earthworm infusion is 50%.

Keywords: Antimicrobial; Earthworm; Infusion

АБСТРАКТ

Диарея - второе по распространенности заболевание в мире после острой респираторной инфекции (ОРВИ). Одной из причин диареи является желудочно-кишечная инфекция, вызванная болезнетворными бактериями (микробами), такими как Salmonella sp., Escherichia coli, Shigella sp. и Vibrio cholerae. Земляной червь Lubricus sp может быть использован в качестве альтернативного лекарственного средства, которое, как считается, способно лечить такие заболевания пищеварительной системы, как тиф, дизентерия и другие расстройства пищеварения, например гастрит. Данное исследование является лабораторным экспериментальным. Исследование проводилось в микробиологической лаборатории медицинского факультета Университета Мухаммадии Джокьякарты (Universitas Muhammadiyah Yogyakarta). Антибактериальная активность измерялась путем определения минимальной ингибирующей концентрации (MIC) и минимальной бактерицидной концентрации (MBC) настоя дождевого червя (Lumbricus rubellus) с помощью теста разведения в пробирке. В данном исследовании использовались бактерии Escherichia coli, Shigella dysentriae и Vibrio cholerae, выделенные из фекалий больных диареей в Мутиланской региональной больнице общего профиля. Цель данного исследования - изучить антибактериальную активность настоя земляного червя (Lumbricus rubellus) в отношении нескольких бактерий, которые обычно вызывают диарею. Согласно результатам исследования, средняя минимальная ингибирующая концентрация (МИК) настоя дождевого червя (Lumbricus rubellus) против кишечной палочки, Shigella dysentriae и Vibrio cholerae составляет более 50 %. Что касается Shigella dysentriae, то среднее значение MBC настоя земляного червя составляет 50%.

Ключевые слова: Антимикробный препарат; инфузория дождевого червя

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INTRODUCTION

Diarrhea is the world's second-largest disease, following Acute Respiratory Infection.[1,2] Generally, diarrhea infection occurs in developing countries and causes death for 3 million people annually.[3] The 2018 Riskesdas showed that the diarrhea prevalence based on the diagnosis of medical workers is 6.8%, while the diarrhea prevalence based on the diagnosis of medical workers and its symptoms is 8,0%.[4] Diarrhea kills around 525,000 children under 5 per year, according to WHO. Children with malnutrition or immune disorders and HIV are at risk of diarrhea.[5]

Naturally traditional medicine can be used as traditional therapy to cure disease. It prevents the occurrence of resistance toward uncontrolled antibiotics. Thus, the development of a naturally traditional medicine business is conducted. The development of this medicine is considered more safe due to better tolerance than antibiotic therapy.[6]

Earthworm (Lumbricus rubellus) is a traditional remedy. The Lumbricus rubellus worm has 76% protein, 10% fat, 1% phosphor, and 0.55% calcium. Worm extract contains anti-purine, antipyretics, anti-dot, arachidonic acid, vitamin, and ascorbate acid to reduce infection-related body temperature. Enzymes include peroxidase, lumbrokinase, catalase, cellulose, phosphatase, lysozyme, and glucuronide degrade positive gram bacteria's cell walls. Lumbricus rubellus produces extracellular products with cytotoxic, anti-bacteria, and phagocytosis characteristics to inhibit harmful microorganisms. Hyaline, amoebocytes, and granular chloragocytes are chemicals.[7]

In 2022, the research conducted by Wahyuni[8], concluded that earthworm extracts of *Pheretima sp and Lumbricus rubellus* have resisting efficacy toward *Staphylococcus aureus and Salmonella typhi*. It is indicated by the anti-bacterial effectiveness of worms *(Lumbricus sp.)*. Therefore, the research is necessarily conducted regarding the anti-bacterial infuse activity of worms *(Lumbricus sp.)* toward some bacterium causing diarrhea, such as *Escherichia coli, Shigella sp.,* and *Vibrio cholerae.*[10]

MATERIAL AND METHODS

A number of instruments used in this study included 10 cm-diameter Petri dishes, sterile loops, measuring pipettes, micropipettes, pipet balls, Memmert ovens, Memmert incubators, Jericho JE-350A autoclaves, infusion pots, blenders, thermometers, and water baths. Erlenmeyer flask, electric stove, tube reaction, spirit, tube rack, sterile cotton, vortex, and label paper. The materials used in this research were McConkey agar media, Kligler's Iron Agar (KIA) medium, Trypticase Soy Agar (TSA) medium, Thiosulfate-Citrate-Bile Salts-Sucrose Agar (TCBS) medium, Brain Heart Infusion (BHI) medium, Brown III standard solution, physiological NaCl solution, sterile distilled water, and earthworm infusion with 100% concentration. The research samples were collected from the feces of diarrhea patients at Muntilan General Hospital, whereas the research subject was earthworms of the *Lumbricus* sp. This study examined *Escherichia coli*, *Shigella sp*., and *Vibrio cholerae*, which were isolated from the feces of diarrhea patients.

The research started by preparing the infusion of earthworm from 75 grams of *Lumbricus sp*. Firstly, the earthworms were cleaned and blended for 10 (ten) minutes until infusion was formed. Subsequently, the infusion was put into the Erlenmeyer flask and sterilized at 90^{o}C for 15 (fifteen) minutes. After being heated, the sterilization was tested. If there was no turbidity in the seed tube, the earthworm infusion could be declared sterile.

The bacterial testing started with isolating and identifying bacteria from the fecal samples. The samples, collected from the diarrhea patients, were diluted in the physiological NaCl solution. After that, the samples were cultured to isolate diarrhea bacteria and planted on McConkey and TCBS media. On the McConkey medium, the colonies with characteristics of large macroscopic

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colonies, red in color, convex, and slightly wet, were the *Escherichia coli* colonies. At the same time, the smaller and transparent colonies planted on the KIA medium were tested for biochemical. The biochemical test had these results, namely Slank (alkaline), Butt (acidic), Gas (-), and H2S (-). This result indicated that the colonies were *Shigella sp*. On the TCBS medium, the greenish-yellow bacteria colony was *Vibrio cholerae*. Three types of bacteria, identified and isolated from the feces, were then smeared on different media. *The first one, Escherichia coli*, was on McConkey, *Shigella sp*. on TSA, and *Vibrio cholerae* on TCBS. All bacteria were subsequently incubated at 37^{o}C for 18-24 hours. Each test bacterium was suspended in 10 ml of physiological NaCl solution. The bacterial suspension was diluted in BHI medium with sterile distilled water until turbidity reached the Brown III standard (108 CFU/ml). Later, the solution was diluted 1:100 using a BHI medium to reduce the bacterial concentration to 106 CFU/ml.

A series of tube dilution tests were carried out to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values of the earthworm infusion against test bacteria. Firstly, 90 (ninety) 5 ml sterile test tubes were prepared. The test took 9 (nine) series with three repetitions; each dilution test needed 10 (ten) tubes. The test tubes were marked 1 to 9, while the others were marked K- (infusion control) and K+ (bacterial suspension control). After that, 1 ml sterile distilled water was added into tubes 2 to 9 and two control tubes. As much as 1 ml of 100% earthworm infusion was later added to tubes 1 and 2, resulting in tube 2's infusion concentration being 50%. The tubes were shaken until homogenous. Subsequently, 1 ml infusion from Tube 2 was taken and injected into Tube 3; the concentration was shaken until homogenous. This stage was repeated to reach the dilution series: Tube 1 100%, Tube 2 50%, Tube 3 50%, Tube 4 12.5%, Tube 5 6.50%, Tube 6 3.150%, Tube 7 1.563%, Tube 8 0.781%, Tube 9 0.391%; Tube 10 contained the remaining of the infusion dilution. Afterward, 1 ml bacterial suspension, which was prepared earlier, was injected into tubes 1 to 11, excluding tube number 10. Tube 10 only contained the remaining dilution and BHI without bacteria; this tube was the material sterility control (negative control). In contrast, Tube 11 had a BHI medium with bacteria and was used as the bacterial growth control (positive control). The final concentrations of the earthworm infusion after being injected with the test bacteria were: 50% for Tube 1; 50% for Tube 2; 12.5% for Tube 3; 6.50% for Tube 4; 3.150% for Tube 5; 1.563% for Tube 6; 0.781% for Tube 7; 0.391% for Tube 8; and 0.195% for Tube 9. All tubes were incubated for 18-24 hours at 37˚C. Minimum Inhibitory Concentration (MIC) was determined when turbidity does not occur in the lowest concentration. It was also determined by noticing the first tube that turns to clear in the tube series with the lowest concentration. The tube that did not show bacterial growth would be cultured on different agar media, namely McConkey (*Escherichia coli*), TSA (*Shigella sp.*), and TCBS (*Vibrio cholerae*). The culture was incubated at 37^{o}C for 18-24 hours. Meanwhile, Minimum Bactericidal Concentration (MBC) was seen from the absence of bacterial growth on nutrient agar with the lowest concentration.

Experiments were conducted in the laboratory for this study. A table containing the outcomes of the examination was used to present the study data that was acquired. A comparison was made between the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the *Lumbricus sp*. infusion and the bacteria that cause diarrhoea, specifically *Escherichia coli, Shigella sp*., and *Vibrio cholerae*.

RESULT

The results of MIC and MBC of the earthworm (*Lumbricus sp.*) infusion against the diarrhea bacteria are presented below. The identified bacteria that commonly cause diarrhea are *Escherichia coli*, *Shigella sp.*, and *Vibrio cholerae*. In this study, the bacteria were

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collected from the patient's feces; information regarding bacteria is presented in Table 1.

Table 1. Identification of fecal isolate bacteria that cause diarrhea

Bacteria	Microscopic Characteristics	Macroscopic Characteristics
Escherichia coli	Stem, red, dispersed	On Mc Conkey: large colonies, red, vortex, slightly wet
Shigella sp.	`Stem, red, dispersed	• On McConkey: small colonies, transparent • On KIA: Slank (alkaline), Butt (acidic), Gas (-), H2S (-)
Vibrio cholerae	Stem, red, dispersed	On TCBS: slightly larger colonies, greenish-yellow, rather dry

From three repetitions of the treatment of each bacterium, the average results of MIC and MBC of the infusion of earthworms (*Lumbricus sp.)* against *Escherichia coli*, *Shigella sp.*, and *Vibrio cholerae* are presented as follows.

Table 2. Results of Minimum Bactericidal Concentration (MBC) and Minimum Inhibitory Concentration (MIC) of the Infusion of Earthworms (*Lumbricus sp.*) against *Escherichia coli* Fecal Isolate Bacteria

No.	*Escherichia coli*	
	MIC (%)	MBC (%)
1.	> 50	> 50
2.	> 50	> 50
3.	> 50	> 50
Average	> 50	> 50

The earthworm infusion (Lumbricus sp.) has MIC and MBC values greater than 50% when it comes to Escherichia coli, according to data in Table 2. It suggests that there is no antibacterial activity of the earthworm infusion against Escherichia coli.

Table 3. Results of MIC and MBC of the Infusion of Earthworms (*Lumbricus sp.*) against *Shigella sp.* Fecal Isolate Bacteria

No.	*Shigella sp.*	
	MIC (%)	MBC (%)
1.	0,098	50
2.	0,098	50
3.	0,098	> 50
Average	0,098	50

Based on the data above, the earthworm infusion (*Lumbricus sp.*) had a MIC value of 0.098% and an MBC value of 50%. It indicates that the earthworm infusion is bactericidal against *Shigella sp.*

Table 4. Results of MIC and MBC of the Infusion of Earthworms (*Lumbricus sp.*) against *Vibrio cholerae* Fecal Isolate Bacteria

No.	*Vibrio cholerae*	
	MIC (%)	MBC (%)
1.	> 50	> 50
2.	> 50	> 50
3.	> 50	> 50
Average	> 50	> 50

The table above shows that more than 50% earthworm infusion (*Lumbricus sp.*) is needed to fight *Vibrio cholerae*. In other words, the *Lumbricus sp.* infusion does not have antibacterial activity against *Vibrio cholerae*. The average value of MIC and MBC of the earthworm infusion (*Lumbricus sp.*) against the three isolated fecal bacteria is presented in Table 5.

Table 5. Results of Average MIC and MBC of the earthworm infusion (*Lumbricus sp.)* against various fecal isolates of diarrhea patients

No	Test Bacteria	MIC (%)	MBC (%)
1.	*Escherichia coli*	> 50	> 50
2.	*Shigella sp.*	0,098	50
3.	*Vibrio cholerae*	> 50	> 50

Based on the data above, it can be concluded that the infusion of *Lumbricus sp.* has the lowest MIC and MBC against *Shigella sp.*; however, it does not have substantial antibacterial activity against *Escherichia coli* and *Vibrio cholerae*.

DISCUSSION

The determination of the Minimum Inhibitory Level (KHM) and Minimum Killing Level (KBM) can be achieved by the dilution method for measuring the antibacterial activity of in vitro materials. Bacterial

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inhibition or death potential decreases with increasing KHM and KBM values of antibacterial materials.

The results of this research show that earthworm infuse (*Lumbricus sp*.) did have neither bactericide nor bacteriostatic toward various bacterium causing diarrhea, mainly *Escherichia coli* and *Vibrio cholera*, where the value of the KHM and KBM was more than 50%. Meanwhile, *Shigella sp.* bacteria toward earthworm infuse *(Lumbricus sp.)* had bactericide properties of 50%.

Earthworm extract contained anti-purine, anti-dot, vitamin, arachidonic acid, and antipyretic substances. These drugs reduced infection-induced body temperature rise. Positive and negative Gramme bacteria, which were found in earthworms, were able to be inhibited by the bioactive components of Lumbricin[1], which were of the peptide class and had a broad spectrum of activity against microbes. Because the earthworms altered the mechanism of membrane permeability by creating pores in the cell wall of the bacterium, the activities that took place within the bacteria's cell were stifled. This was due to the fact that the cytoplasm was exposed to the external environment, which resulted in the cell being lysed. The mechanism of immunity from earthworm could inhibit pathogen bacteria by a means of producing hyaline, and *amoebocytes* granular, which had efficacy in the process of phagocytosis and *chloragocytes*. It could produce extracellular products, which was cytotoxic and antibacterial.[7,8] Also, the earthworm resulted in lysozyme enzyme, which was significant to protect from pathogen microbe attacks. Furthermore, it produces enzymes, such as phosphatase, glucuronides, and peroxidase.

The results of this research demonstrates that the results of earthworm infuse (*Lumbricus sp.*) did not have bactericide or bacteriostatic properties toward various bacterium causing diarrhea, mainly *Escherichia coli* and *Vibrio* cholera. It was showed with the value KHM and KBM that was more than 50%. Moreover, *Shigella sp.* toward earthworm infuse (*Lumbricus sp.*) had bactericide properties of 50%. Thus, this research was in line with the research conducted by Lilis[7], stating that the extract of earthworm (*Lumbricus sp*.) has bactericide effects toward *Shigella flexneri*, while it has bacteriostatic impact toward *Vibrio cholerae*

CONCLUSION

The conclusion from the research that has been carried out is that infusion of earthworms (Lumbricus sp.) does not have antibacterial activity against Escherichia coli fecal isolate bacteria, and Vibrio cholera fecal isolate bacteria. Meanwhile, earthworm infusion (Lumbricus sp.) has antibacterial activity against the fecal isolate Shigella sp.

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